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Detection of *Cyclospora cayetanensis*, *Cryptosporidium* spp., and *Toxoplasma gondii* on imported leafy green vegetables in Canadian survey



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ABSTRACT

A national survey was performed to determine the prevalence of *Cyclospora cayetanensis*, *Cryptosporidium* spp., and *Toxoplasma gondii* in leafy green vegetables (leafy greens) purchased at retail in Canada. A total of 1171 samples of pre-packaged or bulk leafy greens from domestic (24.25%) and imported (75.75%) sources were collected at retail outlets from 11 Canadian cities between April 2014 and March 2015. The samples were processed by shaking or stomaching in an elution buffer followed by oocyst isolation and concentration. DNA extracted from the wash concentrates was tested for *C. cayetanensis*, *Cryptosporidium* spp., and *T. gondii* using our previously developed and validated 18S rDNA qPCR assay with a universal coccidia primer cocktail and melting curve analysis. Test samples that amplified and had a melting temperature and melt curve shape matching the *C. cayetanensis*, *C. parvum*, *C. hominis*, or *T. gondii* controls were sequenced to verify the presence of target DNA. *Cyclospora cayetanensis* was identified in 3 samples of arugula (USA origin), 1 sample of baby arugula (USA and Mexico), 1 sample of baby spinach (USA), and 1 sample of spring mix (USA). *Toxoplasma gondii* was identified in 3 samples of baby spinach (origin USA or USA and Mexico), and *C. parvum* was identified in 1 sample of arugula (USA) and 1 sample of baby spinach (USA and Mexico). To our knowledge, this is the first finding of *T. gondii* in leafy greens in North America. The results of this survey will be useful for assessing the risk of foodborne parasites on ready-to-eat leafy greens that are available at retail.

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1. Introduction

Numerous outbreaks of foodborne illness associated with protozoan parasites on fresh produce have been reported in recent years. During 2013–2014, over 900 people in the US were infected with *Cyclospora cayetanensis* in foodborne outbreaks linked to imported cilantro and salad mixes (CDC, 2014; Harvey, 2013). *C. cayetanensis* has been linked to sporadic outbreaks of gastrointestinal illness associated with the consumption of leafy greens and berry fruits in North America and Europe for over 20 years (Dixon, 2015; Ortega and Sanchez, 2010). *Toxoplasma gondii* has recently been listed as the second most harmful foodborne pathogen in the US based on the disease burden calculated using the disability-adjusted life year (Scallan et al., 2015). Although the risk of *T. gondii*-contaminated water and meat are well established (Guo et al., 2015; Jones and Dubey, 2010), there are few

Abbreviations: qPCR Quantitative polymerase chain reaction; MCAMelt curve analysis.

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reports of contamination of leafy greens with *T. gondii* oocysts (Lass et al., 2012). Waterborne, person-to-person, and animal-to-human transmission routes for *Cryptosporidium* spp. are commonly recognized, but the parasite is also regularly detected in produce surveys and linked to foodborne outbreaks (Dixon, 2015). Of the three protozoan parasites listed above, *C. cayetanensis* is the most common pathogen that is associated with contaminated fresh produce. Direct or indirect contamination of produce may occur at many points between the farm and consumer, through contaminated soil, manure, irrigation or wash water, equipment, or handlers (Gamble, 2015). The consumption of raw, pre-washed, ready-to-eat leafy greens is increasing and there is increased risk for transmission of foodborne parasites from these products since most post-harvest control measures such as washing or disinfection are aimed at reducing bacterial contamination and are ineffective in removing or inactivating the infective oocyst stage of coccidia including *C. cayetanensis*, *Cryptosporidium* spp., or *T. gondii* (Dixon, 2015; Gajadhar and Allen, 2004; Macpherson and Bidaisee, 2015).

There is no single universally accepted “gold standard” method for the elution, isolation, and detection of multiple species of protozoan oocysts from fresh produce, due to differences in the properties of the various food matrices and parasites of concern. An ISO standard method for the detection and enumeration of *Cryptosporidium* and *Giardia* is now available; however, this method involves immunomagnetic separation and immunofluorescent microscopic detection assays and is only genus-specific with no capability for genetic characterization. There are no such assays currently available for *C. cayetanensis*, so detection is generally performed using standard microscopy and is limited by low sample throughput, and analyst subjectivity and fatigue (Traub and Cuttell, 2015). We have developed and optimized a method for isolation of protozoan oocysts from several types of leafy greens and berry fruits and developed and evaluated a qPCR melt curve analysis (qPCR MCA) assay for use in detecting a variety of protozoan oocysts in produce (Lalonde and Gajadhar, 2016), as well as human fecal samples (Lalonde and Gajadhar, 2011; Lalonde et al., 2013). The objective of this study was to estimate the incidence of *C. cayetanensis*, *T. gondii*, and *Cryptosporidium* spp. contamination in leafy greens available at retail outlets in Canada, using this validated qPCR MCA assay.

2. Materials and methods

2.1. Samples

Pre-packaged or bulk leafy greens were collected as part of a national targeted survey program between April 1, 2014, and March 31, 2015, at retail outlets in 11 Canadian cities (Halifax, St. John, Montreal, Quebec City, Ottawa, Toronto, Winnipeg, Saskatoon, Calgary, Kelowna, and Vancouver). A minimum of 200 g of leafy greens was collected for each sample and transported to the CFIA Saskatoon Laboratory for testing. From collection to testing, samples were maintained at 2–10 °C, and the integrity of the packaging was secured to prevent cross-contamination. The types of samples collected were spinach, spring mix, leaf lettuce, romaine, kale, arugula, chard, collards, dandelion greens, rapini, and various mixes of two or more of these types, but did not include head lettuces (Table 1).

2.2. Sample processing

Previously published methods (Cook et al., 2007, 2006a,b) were adapted, optimized, and validated (Lalonde and Gajadhar, 2016) prior to use in this study. Briefly, 35 ± 0.5 g leafy greens were removed from each sample and washed with a glycine buffer (1 M, pH 5.5) using an orbital shaker or a stomacher (Seward, for romaine, red or green leafy lettuces only) to elute any oocysts present. The resulting wash buffer was concentrated by centrifugation at 2000×g for 15 min, and 2.5–3 ml of the sediment subjected to a flotation procedure using Sheather's sucrose solution (sp.g 1.26) for the isolation of oocysts. DNA extraction was performed as described previously (Lalonde and Gajadhar, 2008, 2016).

Table 1

Summary of the number and type of imported leafy greens tested and confirmed positive for *C. cayetanensis*, *T. gondii*, or *C. parvum*.

Sample type	Number tested	Number positive		
		<i>Cyclospora</i>	<i>Toxoplasma</i>	<i>Cryptosporidium</i>
Arugula/baby arugula	107	4 (3.74%) ^a	0	1 (0.93%)
Kale	44	0 ^a	0	0
Spinach/baby spinach	387	1 (0.26%) ^b	3 (0.78%)	1 (0.26%)
Romaine	113	0 ^b	0	0
Chard	39	0 ^a	0	0
Leaf lettuces ¹	226	0 ^b	0	0
Spring mix	124	1 (0.8%) ^b	0	0
Leafy green mixes ²	91	0 ^b	0	0
Other ³	40	0 ^b	0	0
Total	1171	6 (0.51%)	3 (0.26%)	2 (0.17%)

^{a,b}Means within a column that have different superscripts are significantly different ($P \leq 0.05$). There were no significant differences between number of positives per sample type for *Toxoplasma* or *Cryptosporidium*.

¹ Green or red leaf lettuces.

² Mixes of any two or more of the above leafy green types.

³ Any of dandelion, collards, rapini, escarole, mache.

2.3. Quality control measures

Samples were processed to elute and isolate oocysts within 5 days of receipt at the laboratory, and DNA was extracted from wash suspensions within 3 days of processing. Each sample was assigned a unique laboratory identification number and all sample submission information (including date of collection, date received, retail address, brand, country of origin, sample type, sample fitness) was recorded. Samples were stored in the laboratory at 4 °C (± 3 °C) prior to testing to preserve quality. Sample rejection criteria included conditions such as freezing and spoilage which are known to compromise test performance (unpublished data). All analysts performing the testing had successfully completed a formal training session and proficiency testing to demonstrate competency. The laboratory operated within a formal quality management system and is ISO 17025:2005 accredited. A positive DNA extraction process control consisting of approximately 100 *Eimeria papillata* oocysts and a negative DNA extraction process control consisting of reagents only were extracted with each batch of samples processed.

2.4. qPCR with melt curve analysis

All samples were tested for *C. cayetanensis*, *Cryptosporidium* spp., and *T. gondii* using an 18S rDNA qPCR assay with a universal coccidia primer cocktail and melting curve analysis as described previously (Lalonde and Gajadhar, 2011; Lalonde et al., 2013). A standard curve consisting of DNA extracted from 10^5 *C. parvum* oocysts serially diluted 10-fold to be equivalent to 10^5 , 10^4 , 10^3 , 10^2 , and 10^1 oocysts was included on each plate as a positive control, to provide an estimate of oocyst numbers present in positive samples, and to monitor qPCR efficiency and sensitivity. All DNA test samples were run in duplicate and each plate also included a negative control (PCR reagent only no-template) and the DNA extraction positive (*E. papillata* DNA) and negative process controls described above. Plasmid DNA consisting of the cloned ~315 bp 18S rDNA target region of *C. cayetanensis*, *T. gondii*, *C. hominis*, *Cystoisospora belli*, and *Eimeria bovis* were included on each plate as additional positive controls. The target coccidia were detected by comparing melt curves generated from test samples to those of the coccidia plasmid DNA controls.

2.5. 1.4. Sequence analysis of amplicons

Test samples that amplified with the universal coccidia primers and had a melting temperature and melt curve shape matching the *C. parvum*, *C. hominis*, *T. gondii*, or *C. cayetanensis* controls were sequenced to verify the presence of target DNA. The universal primer cocktail composed of a mixture of all primers was used for sequencing. PCR products were purified using the Qiagen PCR Purification Kit according to the manufacturer's instructions and then sequenced in both directions. Forward and reverse sequence reads were assembled and trimmed using Clone Manager (version 9.3, Sci-Ed Software) and compared to the Genbank collection of nucleotide sequences using BLASTn. The number of positive samples detected in each produce type was compared using a one-way ANOVA with the Tukey's multiple comparison test. Significance was considered when $P \leq 0.05$.

3. Results

A summary of the number and types of leafy greens deemed suitable for testing is provided in Table 1. Of the 1171 samples tested, 284 originated from Canada, 786 from the USA, 87 from USA and Mexico (consisting of product grown and/or processed in both countries), 13 from Mexico, and 1 from the Dominican Republic. *Cyclospora cayetanensis* was detected by qPCR MCA in three samples of arugula (USA origin), one sample of baby arugula (USA and Mexico), one sample of baby spinach (USA), and one sample of spring mix (USA). *Toxoplasma gondii* was detected by qPCR MCA in three samples of baby spinach (origin USA and USA/Mexico). *Cryptosporidium parvum* was detected by qPCR MCA in one sample of arugula (USA) and one sample of baby

Table 2
Summary of information on positive samples tested by qPCR MCA and the parasites detected.

Parasite detected	Leafy green (type)	Month collected	Country of origin	BLASTn match accession no.
<i>C. cayetanensis</i>	Arugula (organic)	October	USA	99% <i>C. cayetanensis</i> AB368541.1
	Arugula (conventional)	October	USA	98% <i>C. cayetanensis</i> AB368541.1
	Arugula (organic)	October	USA	99% <i>C. cayetanensis</i> AB368541.1
	Baby arugula (organic)	November	USA and Mexico	98% <i>C. cayetanensis</i> AB368541.1
	Spring mix (organic)	October	USA	99% <i>C. cayetanensis</i> AB368541.1
	Baby spinach (organic)	October	USA	98% <i>C. cayetanensis</i> AB368541.1
<i>T. gondii</i>	Spinach (organic)	October	USA	99% <i>T. gondii</i> KM875436.1
	Spinach (organic)	October	USA and Mexico	98% <i>T. gondii</i> KM875436.1
	Spinach (conventional)	October	USA	99% <i>T. gondii</i> KM875436.1
				98% <i>C. parvum</i> HQ259583.1
<i>C. parvum</i>	Arugula (conventional)	May	USA	98% <i>C. parvum</i> KP730316.1
	Baby spinach (organic)	May	USA and Mexico	98% <i>C. parvum</i> KP730316.1

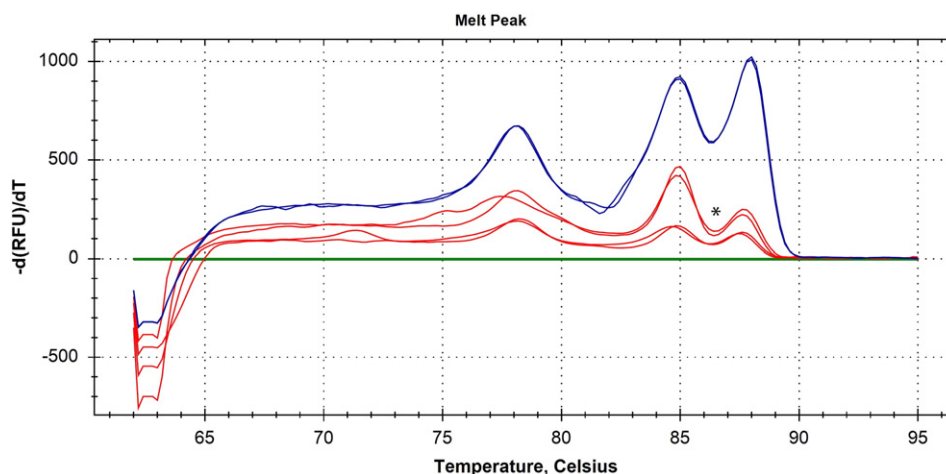


Fig. 1. Graphs of qPCR melt peaks from selected leafy green samples that were confirmed positive for *C. cayetanensis* (red, marked with asterisk) and *C. cayetanensis* plasmid DNA as positive control (blue).

spinach (USA and Mexico). All samples positive for *C. cayetanensis*, *C. parvum*, and *T. gondii* were confirmed by sequencing of the amplicon (Table 2). Figs. 1, 2, and 3 show representative melt curve graphs from selected samples that were positive for *C. cayetanensis*, *C. parvum*, and *T. gondii*.

All of the samples confirmed positive for *C. cayetanensis* and *T. gondii* were collected in October and November and were imported to Canada from USA or USA and Mexico. The samples confirmed positive for *C. parvum* were collected in May and also originated from the USA or USA and Mexico. Of the 11 samples confirmed positive for coccidia in this survey, 8 (73%) were organic and 3 (27%) were conventionally grown leafy greens, according to the package labelling. The most contaminated leafy green type was arugula or baby arugula with 4.7% of samples testing positive for *C. cayetanensis* ($P \leq 0.05$) or *C. parvum*, followed by spinach or baby spinach with 1.3% of samples testing positive for *C. cayetanensis*, *C. parvum*, or *T. gondii*.

4. Discussion

This study achieved its goal of applying a broadly specific screening assay for the detection of protozoan oocyst species of human health concern in a survey of domestic and imported leafy greens available at retail in Canada. Overall, the prevalence of *C. cayetanensis* and *C. parvum* were lower in this study (0.51 and 0.17%, respectively) than in a previous survey of 544 packaged leafy green samples in Ontario, Canada (Dixon et al., 2013) which detected 1.7% and 5.9%, respectively, using PCR and immunofluorescent or fluorescent microscopy. The present survey includes 1171 samples from 11 cities and is therefore more representative of what is available to consumers across Canada. Surveillance studies from around the world report widely varying prevalences for *C. cayetanensis* and *Cryptosporidium* spp. in fresh produce, attributable to differing agriculture practices, water

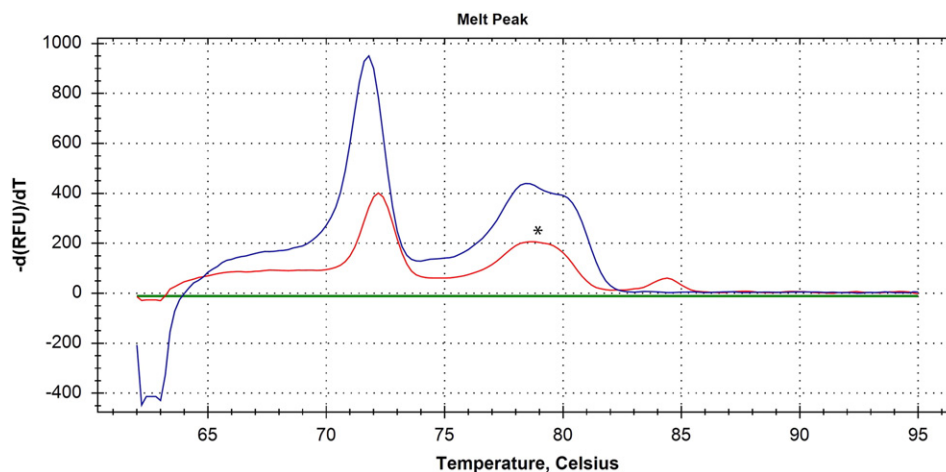


Fig. 2. Graphs of qPCR melt peaks from a selected leafy green sample that was confirmed positive for *C. parvum* (red, marked with asterisk) and *C. parvum* plasmid DNA as positive control (blue).

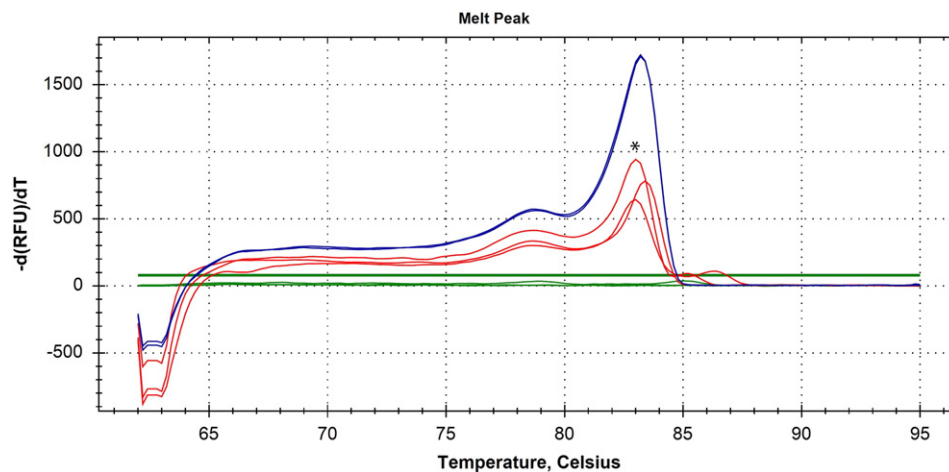


Fig. 3. Graphs of qPCR melt peaks from leafy green samples that were confirmed positive for *T. gondii* (red, marked with asterisk) and *T. gondii* plasmid DNA as positive control (blue).

quality and availability, sanitation, and detection methodologies (Dixon, 2015). The higher overall incidence of coccidia on organic leafy greens in this survey also suggests that certain agricultural practices (such as the use of manure fertilizer) may result in increased contamination. All of the positive samples identified in this study were reported for appropriate regulatory consideration; however, the shelf life of the contaminated products had expired by the time results were reported, and no illness outbreaks are known to be associated with the affected products.

This is currently the only known survey for *Toxoplasma* in leafy greens in Canada, and the first reported evidence of *T. gondii* contamination of fresh produce in North America. Detection of *T. gondii* on leafy vegetables and/or fruits have been reported elsewhere such as in Poland (Lass et al., 2012), Saudi Arabia (Al-Megrm, 2010), and Pakistan (Haq et al., 2014) using microscopy, PCR, or a combination thereof. Reported outbreaks of toxoplasmosis linked to the consumption of contaminated fresh produce are very rare, likely due to mild symptoms or the delayed and varied onset of disease manifestations. Since *T. gondii* can be transmitted from multiple sources, it can be difficult to link positive cases to a common exposure route (Dixon, 2015; Jones and Dubey, 2012). A lack of evidence for *T. gondii* infection linked to produce should not downplay the potential risk. The global distribution of felids, robust survival of oocysts in the environment, and ineffectiveness of many currently used pre- and post-harvest washing and disinfection methods strongly suggest that fresh produce is a generally unrecognized transmission vehicle for *T. gondii* to humans. In addition, testing of fresh leafy greens and berry fruits for *T. gondii* oocysts is not routinely performed as a food safety control measure; this may be due to an underestimation of the potential risk or a lack of validated detection methods or a combination of these. The surveillance data presented here regarding detection of *T. gondii* in leafy greens provides a crucial first step in the identification of potential sources of toxoplasmosis infection and potential risks to consumers.

A limitation of molecular assays for detection of protozoan oocysts in produce is the inability to distinguish live from dead organisms, making it difficult to interpret the results and determine the significance of positive results with respect to the risk posed by the contamination. However, populations of oocysts often consist of both viable and non-viable organisms, and any finding should be considered an indicator of risk. In bacterial detection methods, samples are commonly subjected to an enrichment step prior to detection, so presumably most cells detected are viable or are present in sufficient numbers to permit enumeration and thus represent a food safety risk for consumers. *In vitro* propagation of protozoan oocysts is generally not possible or practical, so detection methods rely on washing to remove and concentrate minute quantities of oocysts from the food matrix followed by detection by microscopy, immunoassay, or PCR. The recovery rate of oocysts from food matrices varies widely and depends on the produce type and condition, wash method, and wash buffer used. Recovery rates have been demonstrated to be between 3 and 18% for leafy greens and berry fruits (Lalonde and Gajadhar, 2016). Thus, even if there was a reliable method for determining if a very small number of recovered oocysts were indeed non-viable and non-infective, these low recovery rates from the produce would make it very difficult to declare any sample as minimal risk, since viable/infected oocysts could still remain on the sample and the distribution and concentration could be highly variable in the batch of produce from which the sample was taken. The qPCR MCA can provide an estimate of oocyst quantity detected and can reliably detect DNA from as few as 10 oocysts (Lalonde and Gajadhar, 2011). The infectious dose for *Cryptosporidium* is estimated at 10 or fewer oocysts (Chappell et al., 2006) and is thought to be equally low for *Toxoplasma* (Dubey et al., 1996; Fayer et al., 2004) and *Cyclospora*. Even a single oocyst contains 8 infective sporozoites and is capable of establishing an infection. Another factor to consider is that the conditions that favor prolonged shelf life in leafy greens, namely cool, moist conditions, also generally favor oocyst survival in the environment. Therefore, a positive PCR result for detection of protozoan oocysts in produce should be considered an indication of risk to public health.

In this survey, *T. gondii* and *C. cayetanensis* were detected in the fall months of October and November, and *C. parvum* during the spring, in May. Outbreaks of cyclosporiasis and cryptosporidiosis in endemic regions are commonly seasonal, most typically

being reported during the summer months (Hall et al., 2012; Painter et al., 2015). However, the peak seasons can vary depending on the regional climate where contamination occurs, as rainfall, runoff, flooding, drought, temperature, and humidity can impact the survival and/or sporulation of oocysts in the environment (Fletcher et al., 2012; Gajadhar et al., 2015). All of the samples found to contain *C. cayetanensis* and *T. gondii* in this study were imported from either the USA or USA and Mexico (package contained a mixture from both countries) where regional climate conditions or pre-/post-harvest practices may have favored oocyst survival and transmission during the fall months. In addition, this survey included 286 samples of domestic leafy greens which were collected and tested during the months they were exclusively available, which was June, July, August, and September due to the relatively short Canadian growing season. Fewer imported samples were tested during these four summer months: 131 (31%) of 417 samples, all of which were from the USA only. Considering the low risk of *C. cayetanensis* transmission from domestic produce, future surveys for this parasite specifically should be designed to target imported produce during the spring and summer months.

The quality of data generated from food safety surveillance studies such as this depends heavily on the selection of a reliable validated method for the detection and the application of appropriate quality assurance measures and controls in the laboratory, including analyst proficiency. The qPCR MCA assay applied here has been demonstrated as fit for use in detecting protozoan oocysts in human feces, leafy greens, and berry fruits (Lalonde and Gajadhar, 2011, 2016; Lalonde et al., 2013). The oocyst isolation method we used was adapted from a previously published method (Cook et al., 2006a,b) which is now being revised as an ISO standard method for detection and enumeration of *Cryptosporidium* and *Giardia* in fresh leafy greens and berry fruits; we optimized and validated the method and use it routinely in our laboratory. The assay is limited by relatively low recovery rates of the oocysts from produce (Lalonde and Gajadhar, 2016), and the inability to determine if any recovered oocysts are viable and infective. The broad specificity of the universal primer cocktail, while useful in targeting multiple species of coccidia, may also result in amplification of non-coccidia species in environmental samples (such as yeast) and thus also reduce the assay sensitivity. During routine testing of leafy greens, non-specific amplicons of fungal origin are observed most commonly in samples collected during the summer months (data not shown). This suggests that maintaining cool storage temperatures for samples prior to testing with qPCR MCA is an important measure for ensuring optimal assay sensitivity. In addition, further molecular characterization (ie species-specific PCR, whole genome or targeted sequencing, other single nucleotide polymorphism-based genotyping assays, etc) of positive samples would be necessary to facilitate molecular epidemiology investigations to link contaminated produce to cases of human infection.

Despite these assay limitations, the results of this survey provide a snapshot of the prevalence of protozoan oocyst species of human health concern in domestic and imported leafy greens in Canada, which may assist in developing food safety guidelines, pre- and post-harvest control measures, and risk analysis. In addition, these findings should be considered in the design and direction of future monitoring surveys of imported leafy greens and other fresh produce, and when providing advice to consumers. Guidelines on foodborne parasites are currently being developed by Codex Alimentarius. Although no protozoan oocysts were detected in domestic leafy greens, we tested fewer of these samples overall. The high number of temporary foreign workers employed in the produce industry from countries where *C. cayetanensis* is endemic, and the widespread incidence of *Cryptosporidium* spp. and *T. gondii* in Canada suggest that domestic leafy greens are not of negligible risk for parasites.

Conflict of interest

The authors have no conflict of interest.

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